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Butanol production from wheat straw by simultaneous saccharification and fermentation using *Clostridium beijerinckii*: Part II—Fed-batch fermentation

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ABSTRACT

In these studies, *Clostridium beijerinckii* P260 was used to produce butanol (acetone–butanol–ethanol, or ABE) from wheat straw (WS) hydrolysate in a fed-batch reactor. It has been demonstrated that simultaneous hydrolysis of WS to achieve 100% hydrolysis to simple sugars (to the extent achievable under present conditions) and fermentation to butanol is possible. In addition to WS, the reactor was fed with a sugar solution containing glucose, xylose, arabinose, galactose, and mannose. The culture utilized all of the above sugars. It was noticed that near the end of fermentation (286–533 h), the culture had difficulties utilizing xylose. As a result of supplemental sugar feed to the reactor, ABE productivity was improved by 16% as compared with previous studies. In our previous experiment on simultaneous saccharification of WS and fermentation to butanol, a productivity of 0.31 $g L^{-1} h^{-1}$ was observed, while in the present studies a productivity of 0.36 $g L^{-1} h^{-1}$ was observed. It should be noted that a productivity of 0.77 $g L^{-1} h^{-1}$ was observed when the culture was highly active. The fed-batch fermentation was operated for 533 h. It should be noted that *C. beijerinckii* P260 can be used to produce butanol from WS in integrated fermentations.

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1. Introduction

The importance and history of butanol (acetone–butanol–ethanol; AB or ABE) production by fermentation has been described previously [1]. Butanol is a cleaner and superior fuel extender/oxygenate than ethanol [2,3] with octane numbers 113 and 94 as compared with that of 111 and 94 for ethanol [4]. With these superior fuel properties, and recent advances in biotechnology and bioprocessing (development of superior strains and advanced process technology) commercial interest has returned to butanol fermentation [2,5–8]. We have been successful in identifying some of the novel unit operations such as fermentation of concentrated substrates, upstream and downstream processing used in the production of butanol. In addition to development of superior microbial strains and novel process technology, substrate cost plays an important role in the commercial production of butanol [9,10]. As a result, it has been identified that wheat straw (WS) could be a successful industrial substrate for butanol production [1,11].

According to the Food and Agriculture Organization of the United Nations, approximately 616×10^6 tonnes of wheat was

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produced worldwide, including 51.0×10^6 tonnes in the USA in 2006 [12]. It is estimated that approximately 1.85×10^9 tonnes of WS was produced. WS is composed of 35–45% cellulose, 20–30% hemicellulose, and a relatively low lignin content (<20%) [13]. The low lignin content makes its bioconversion to biofuels particularly attractive. Use of WS as a potential substrate has been increasingly studied for various bioconversion processes [14–16]. In the current study, the conversion of WS to ABE was investigated.

There are a number of processes that can be employed to pretreat WS including acid, alkali, and ammonia fiber expansion. Over the recent years, dilute acid has been seen as a preferred method for pretreatment as it results in a highly digestible substrate for further enzymatic hydrolysis of cellulose with reasonably high sugar yields from hemicellulose [17,18]. As described in our previous work (Process V—Conclusions section) on simultaneous saccharification and fermentation to butanol [1], the rate of sugar utilization was greater than the rate of hydrolysis of WS, thus leaving the culture limited for sugars, which may have affected the fermentation adversely, thus reducing productivity. Also, WS was not hydrolyzed completely. The objectives of the present studies were 3-fold: (i) to examine whether our previous fermentation was impacted by sugar limitation, (ii) to improve WS hydrolysis to 100%, and (iii) to operate simultaneous saccharification and fermentation until culture ceases producing AB/ABE. These studies are considered important as achieving the above objectives would improve bioconversion efficiency of WS to butanol, making its production via this route economically attractive compared with when glucose is used. It should be noted that there has been a strong interest in reviving butanol fermentation as numerous industries are taking keen interest in this valuable fermentation including DuPont (USA) and British Petroleum (UK) [1].

2. Materials and methods

2.1. Culture and cell propagation

Clostridium beijerinckii P260 was a generous gift from Professor David Jones (University of Otago, Dunedin, New Zealand). The details of culture maintenance and cell propagation have been published in previous paper [1]. WS hydrolysate (WSH) medium (1 L) was inoculated with 70 mL of actively growing culture in P2 medium [1]. WS was pretreated with dilute acid (1% v/v) as described below.

2.2. Wheat straw (WS)

WS was obtained from a local farmer (irrigated location; Mannito, IL, USA). The cultivars of wheat were FS 634 and FS 645 (FS—Farm Service). Wheat (winter wheat) was harvested in early to late June 2003 and WS was baled and stored dry until used. The composition of WS has been given elsewhere [13].

2.3. Preparation of WSH

Eighty-six grams of dry WS was placed in a 2 L beaker. To the WS, 1 L of dilute sulfuric acid solution (10 mL sulfuric acid in 990 mL water) was added, followed by autoclaving at 121 °C for 60 min. The WS suspension was allowed to cool to room temperature. The pH of this suspension was 1.5. Followed by cooling, the mixture was transferred to a sterile 2.5 L glass fermentor (Bioflo 2000; New Brunswick Scientific Co., New Brunswick, NJ, USA). The pH of the mixture was adjusted to approximately 6.5 with 10 M NaOH solution. To 1 L medium, 10 mL of 100 g L^{-1} yeast extract solution and 10 mL each of P2 stock solutions (vitamins, minerals, and buffer) were added. At this stage, the mixture was sparged with approximately 100 mL min^{-1} oxygen-free N_2 gas and agitated at 150 rpm for 24 h to create anaerobic conditions. To the fermentation medium, 6 mL each of the three enzymes (Celluclast 1.5 L (cellulase; supplier—Sigma Chemicals, St. Louis, MO, USA), Novozyme 188 (β-glucosidase; supplier—Sigma Chemicals), and Viscostar 150 L (xylanase; supplier—Dyadic Corporation, Jupiter, Florida, USA)) and actively growing culture (70 mL) were added. Prior to inoculation with the culture, agitation and N_2 sparging was stopped. Fermentation temperature was controlled at 35 °C by circulating water through the cooling coil provided with the fermentor using a Polystat® heated circulating water bath (Cole-Parmer, Vernon Hills, IL, USA).

2.4. Enzyme inhibition by sugar

In order to study whether supplementation of sugar (5–10 g L^{-1}) would inhibit hydrolytic enzymes during saccharification, 8.6 g of WS was pretreated with 100 mL dilute (10 mL H_2SO_4 in 990 mL distilled water) sulfuric acid as described previously [19] and in Section 2.3. After cooling and adjusting the pH to 5.0 with 10 M NaOH, 0.6 mL of each of the three hydrolytic enzymes was added. Prior to incubation at 45 °C, 0, 5, and 10 g L^{-1} (0, 0.5, and 1.0 g) glucose was added to the three bottles. The mixture was then incubated with agitation at 80 rpm. Samples were taken at regular intervals for sugar analysis by HPLC.

2.5. Fermentation

Fermentation experiments were carried out in a pH-controlled 2.5 L bioreactor (Bioflo 2000™, New Brunswick) under anaerobic conditions. After inoculation, oxygen-free N_2 gas was swept across the surface of the medium to maintain an anaerobic environment. The pH was controlled with a 4 M KOH solution to 6.5. Antifoam (Antifoam 204, Sigma Chemicals) was used to control foaming. An initial sample (0 h) was taken immediately following the addition of enzymes for sugar analysis. Regular samples were taken to determine butanol/ABE production and sugar utilization, and to control the fermentation.

After 24 h of inoculation (when 5–7 g L^{-1} ABE accumulated), gas stripping was initiated by circulating CO_2 and H_2 gases (produced during fermentation) at a rate of 4 L min^{-1}. Gas was bubbled (4 L min^{-1}) to agitate the culture and recover butanol from the broth. Also at this time, a sugar feed was started to the fermentor at a flow rate of 6 mL h^{-1} (0.1 mL min^{-1}), which was changed as needed. The first sugar feed consisted of (1480 mL, Feed I) 105 g glucose, 75.6 g xylose, 18.6 g arabinose, 12.8 g galactose, and 10.0 g mannose in distilled water. The feed was sterilized at 121 °C for 15 min. When Feed I was completely utilized, a second feed (760 mL, Feed II) containing

74.1 g glucose, 53.5 g xylose, 13.2 g arabinose, 9.0 g galactose, and 7.1 g mannose was prepared. These amounts of sugars were selected to mimic their proportion in WS. Addition of the sugar feed was done to raise the level of sugar in the fermentor. During the fermentation, stock solutions (4 mL each of vitamin, mineral, and buffer) and yeast extract (10 mL of 40 $g L^{-1}$) were added periodically to aid the fermentation. When needed, sterile distilled water was also added to keep a constant level in the fermentor.

ABE vapors (in stripping gas) were condensed using a Thermo Haake Phoenix Circulator (Thermo Haake, Newington, NH, USA). The temperature of coolant in the machine was controlled at 1 °C. The condensate was collected in a receiver. Condensate was then transferred to 120 mL screw-cap bottles and stored at 4 °C until ready for analysis. Samples from the bioreactor were stored in a −20 °C freezer until ready for analysis by HPLC (for sugars) and GC (for ABE and acids).

After 120 h of fermentation, 50 mL of fermentation broth was removed from the reactor and transferred into a 100 mL bottle. The bottle was then placed in an incubator at 45 °C for 3 days to check whether hydrolysis was complete. Samples at 0 time (start of incubation) and 3 days were taken to analyze for sugars.

2.6. *Analyses*

Fermentation products (ABE, acetic acid, and butyric acid) and sugars were analyzed as described in a previous paper [1]. ABE productivity was calculated as total ABE (present in the reactor plus condensed) produced in $g L^{-1}$ divided by the fermentation time and is expressed as $g L^{-1} h^{-1}$. ABE yield was calculated as described elsewhere [1]. During this experiment, cell concentration was not measured as suspended solids interfered with optical density measurement.

3. Results and discussion

Initially, three experiments were conducted to find out whether supplementation with sugar (5–10 $g L^{-1}$) would inhibit hydrolysis of WS. The results of hydrolysis are shown in Fig. 1. During the initial 24 h of the hydrolysis period, hydrolysis was fast, which slowed down considerably during the next 48–72 h. However, it should be noticed that hydrolysis continued during this period, though at a slow rate. The enzymes were able to hydrolyze WS when supplemented with 5–10 $g L^{-1}$ glucose without inhibition (total sugar 23.3–58.5 $g L^{-1}$ due to sugars released during pretreatment, presence of 5–10 $g L^{-1}$ added glucose, and sugars released as a result of enzymatic hydrolysis). These studies were performed as we planned to feed our fed-batch reactor (operated for simultaneous saccharification and fermentation) with a sugar solution to keep a non-inhibitory (both for enzymatic hydrolysis and fermentation) level of sugar ($<60 g L^{-1}$) in the bioreactor.

In order to improve ABE productivity, batch fermentation was initiated with 86 $g L^{-1}$ WS. As sugars were used by the culture, a feed containing sugar solution was initiated (to the reactor), thus operating the reactor in a fed-batch mode. The amounts of sugars that were present in Feed I (sugar concentration 150 $g L^{-1}$) and II (sugar concentration 206.4 $g L^{-1}$) and media volumes have been given in Section 2. The feed was regulated to control a sugar concentration in the range of 1.6–42.5 $g L^{-1}$ (preferably $<35 g L^{-1}$) to keep the culture growing without any substrate inhibition. The studies reported in Fig. 1 have demonstrated that this level of sugar was not inhibitory to enzymatic hydrolysis.

Since one of the objectives of these studies was to hydrolyze WS completely (to the extent achievable using these commercial enzymes under present conditions; pH, temperature, concentration), after 120 h of saccharification and fermentation it was confirmed that the hydrolysis of WS was complete. After this, fermentation was continued to study the length of fermentation until the culture stopped fermentation. The concentrations of ABE that were achieved during the fermentation are presented in Fig. 2A. During the fermentation, acetone and butanol concentrations fluctuated. Oscillations in acetone and butanol concentrations are characteristic of this fermentation and have been reported

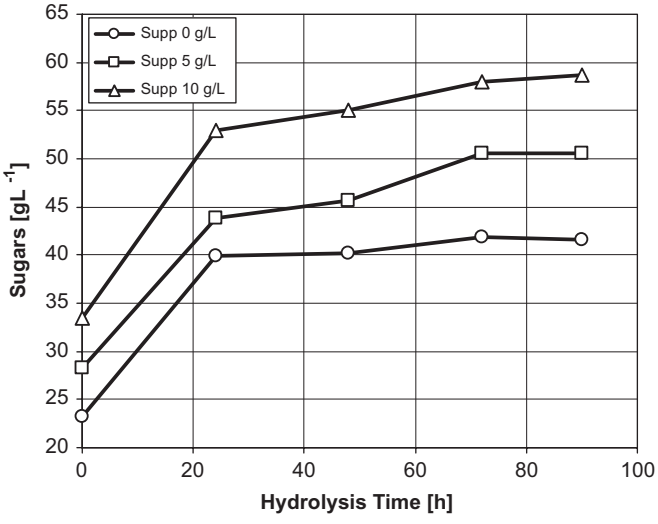

Fig. 1 – Hydrolysis of WS in the presence of sugars using enzymes (cellulase, β-glucosidase, and xylanase).

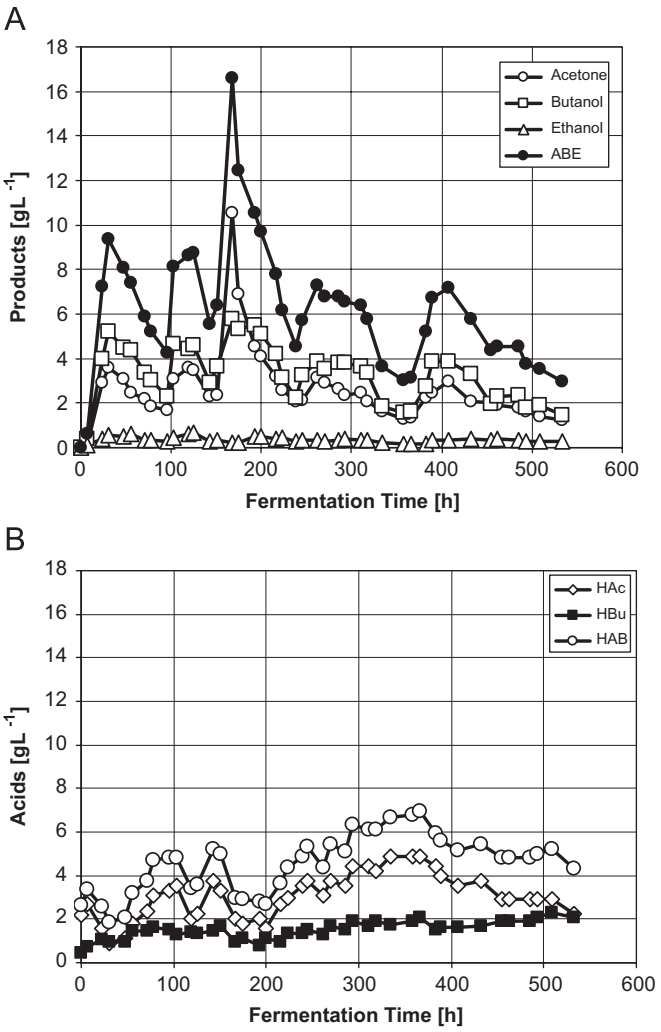

Fig. 2 – Simultaneous saccharification and fermentation of WS to ABE in a fed-batch reactor of *C. beijerinckii* P260. (A) ABE vs. fermentation time; (B) total acids in the reactor at various times. Hac—acetic acid, HBu—butyric acid, HAB—total acids.

previously [20–24]. Fluctuations occur due to inhibition caused by the products. In the reactor, maximum ABE concentration of 16.59 g L^{-1} was observed at 167 h (acetone 10.57, butanol 5.80, ethanol 0.22 g L^{-1}). As can be seen in Fig. 2B, the levels of acids fluctuated significantly, in particular acetic acid. At the end of fermentation, the total concentration of acids in the reactor was 4.3 g L^{-1} (Fig. 2B). Although attempts were made to control a constant level of total sugar (in the reactor), their concentration inside the reactor fluctuated depending on the metabolism of the culture. It was noticed that at the end of 286 h, 47.9 g L^{-1} sugars had accumulated in the reactor (Fig. 3). After 270 h, the culture experienced difficulty in metabolizing xylose efficiently, which accumulated to 27.1 g L^{-1} (Fig. 3A). In order to force the culture to more completely metabolize xylose, the feed rate was reduced. By the end of 533 h, the culture used all the sugars except 3.89 g L^{-1} xylose. However, during the last stages of fermentation, xylose metabolism was significantly reduced.

During these studies, the presence of acetic and butyric acids was observed in the recovered product stream. In this experiment, 0.02–0.67 g L^{-1} acetic acid was measured in the condensate. Butyric acid concentration was 0.02–0.8 g L^{-1}. The total amount of acids varied from 0.09 to 1.47 g L^{-1} (Fig. 4). In our previous work [2,25,26], no acids were measured in the product stream. As described by Hamer [27], the humidity of recycled gas may have played an important role in removing acids from the broth. Recovery of ABE from the condensate containing acids would require distillation.

In these studies, an overall ABE productivity of 0.36 g L^{-1} h^{-1} was achieved. During the run, 378.9 g of total sugar was fed to the reactor in addition to 86 g of WS. In our previous experiments on hydrolysis of WS, 86 g of WS resulted in 55 g of total monomeric sugars [11,19]. Summation of the sugars obtained from WS and fed to the reactor resulted in 433.9 g of total sugar. At the end of fermentation, 3.89 g (in 1 L medium) sugar (as xylose) was left unutilized. Thus, 430 g of total sugar was used by the culture. In the system, 192.0 g ABE was produced, resulting in a solvent yield of 0.44. As a result of feeding the reactor with a sugar solution, ABE productivity was improved from 0.31 to 0.36 g L^{-1} h^{-1}, which is an increase of 16%. This suggested that our previous fermentation [1] was

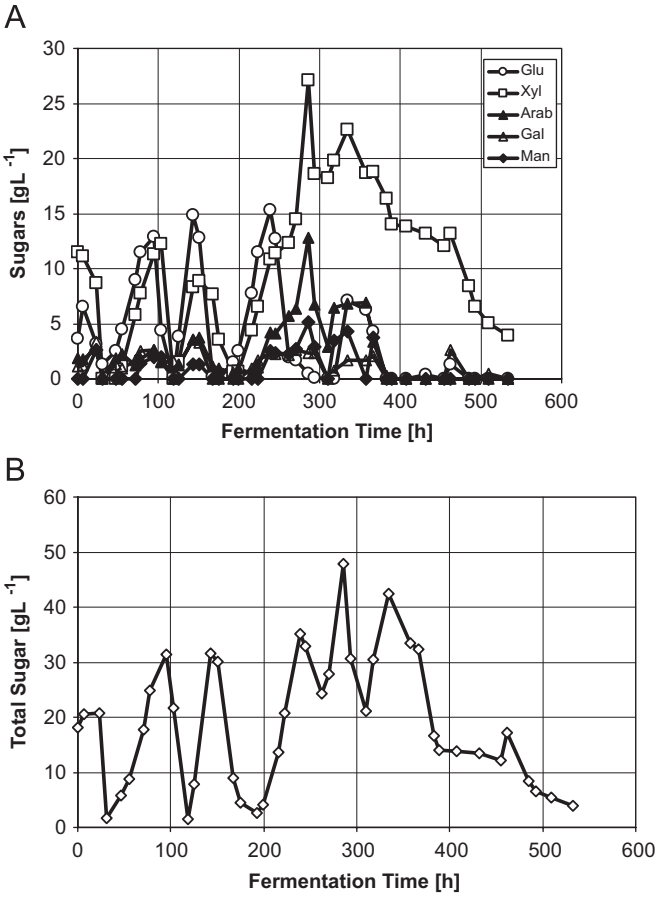

Fig. 3 – Sugar concentrations in the reactor during ABE production using *C. beijerinckii* P260. (A) Various sugars vs. fermentation time; (B) total sugar concentration vs. fermentation time.

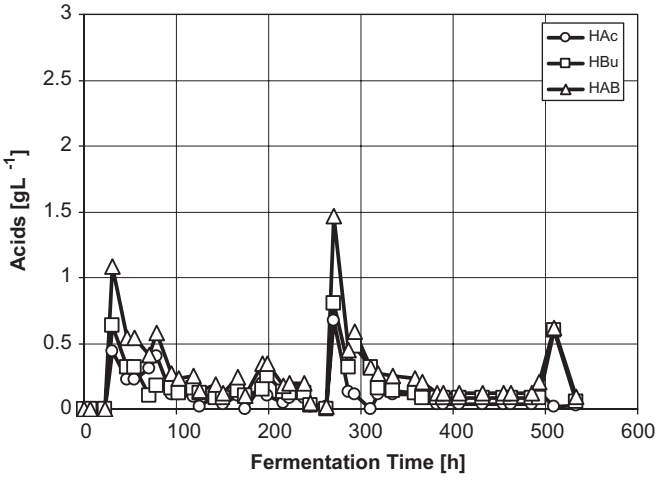

Fig. 4 – Presence of acids in recovered stream (condensate). Hac—acetic acid, HBu—butyric acid, HAB—total acids.

affected negatively due to lack of sugars, resulting in low productivity.

As WS was hydrolyzed completely (to the extent achievable with commercial enzymes), this made simultaneous saccharification and fermentation a successful experiment. The experiment was run for 533 h and during this time, ABE was removed continuously using gas stripping. Depending on the metabolism of the culture and the presence of sugar in the fermentation broth, the sugar feed rate ranged from 0 to 14 mL h^{-1} (Fig. 5A). A feed rate of 14 mL h^{-1} was regulated when the culture was highly active (143–175 h), which was obvious from the vigorous gas production by the culture. As acetone, butanol, and acid concentrations fluctuated, the feed rate was regulated to the reactor accordingly. ABE

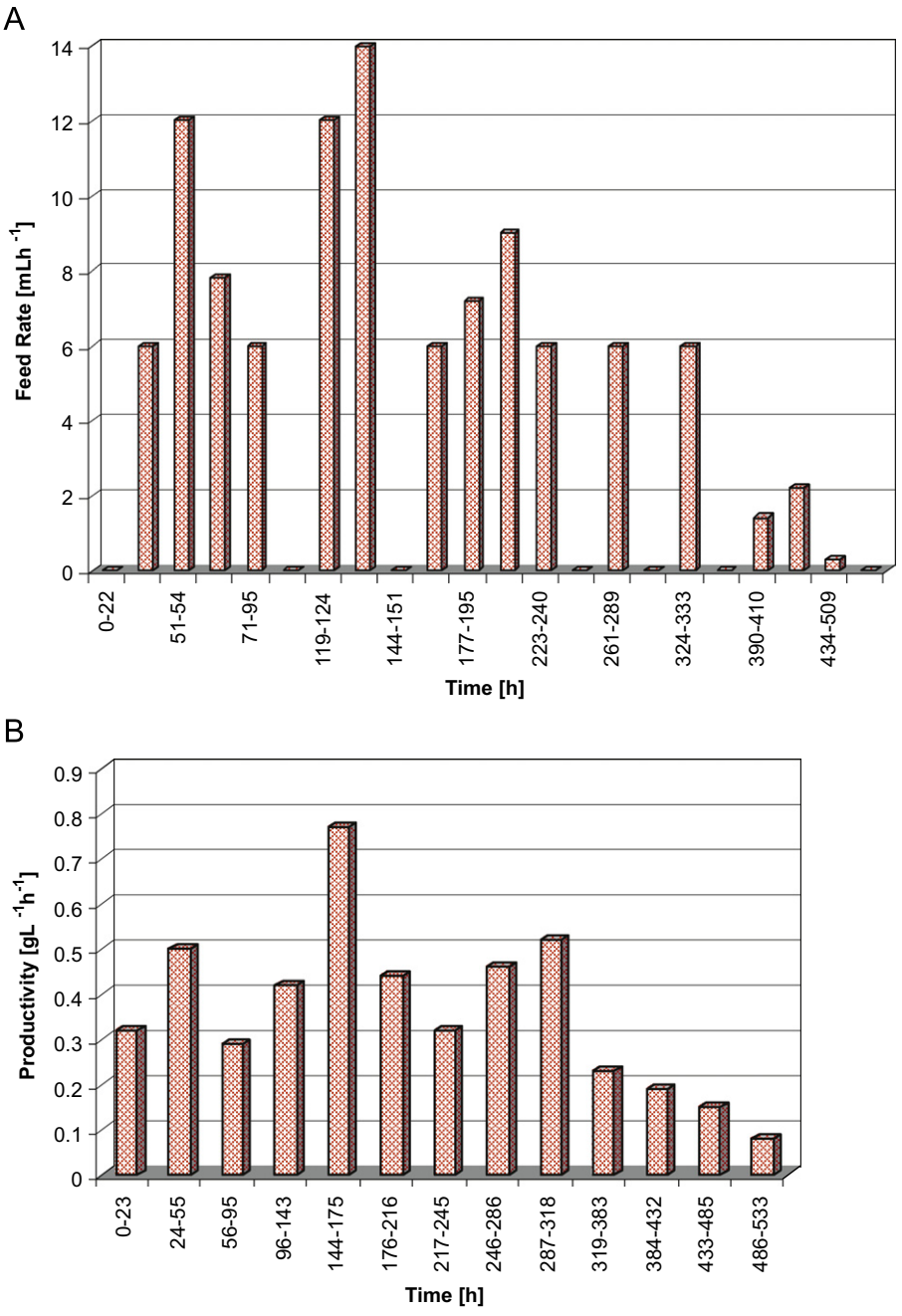

Fig. 5 – Fermentation parameters during ABE production. (A) Sugar feed rate to the reactor at various times; (B) ABE productivities.

concentration in the reactor, varied sugar levels, and different feed rates resulted in fluctuating ABE productivities (Fig. 5B). Between 144 and 175 h a productivity of 0.77 $g L^{-1} h^{-1}$ was observed. This suggests that the culture should be monitored in this state to achieve a high productivity during simultaneous fermentation and saccharification. In order to avoid deficiency of nutrients to the culture, 4 mL of each of the three stock solutions and 10 mL of yeast extract solution (40 $g L^{-1}$) were injected into the reactor at 52, 103, 123, 206, 290, 337, 410, and 512 h.

At this stage, we are not clear as to why xylose utilization was reduced (after 270 h). It is viewed that further experiments be carried out to find out the reason for cessation of xylose utilization. In our previous studies on the production of ABE from glucose using *C. beijerinckii* BA101, the culture stopped using glucose after 201 h of fermentation [26]. During these studies, 500 g glucose was utilized and 232.8 g ABE was produced. In the present studies 430 g of mixed sugars, including 55 g from WS (86 g WS), was utilized. A comparison of the present system with a glucose-based fed-batch system [26] demonstrates that fed-batch butanol fermentations without bleed often become acidogenic after 8–11 days of operation. The exact reasons of this cause have not been identified. It is speculated that decreased water activity

and/or accumulation of unknown toxic compounds may have stopped solventogenesis. It has been observed that some of the hydrolysates of agricultural residues (such as corn fiber) contain inhibitory chemicals that affect/inhibit ABE fermentation negatively [28]. The inhibitors that are generated during hydrolysis of agricultural residues include salts (as a result of neutralization), syringaldehyde, acids (glucuronic, coumaric, and ferulic acids), etc. [28]. In order to estimate the extent of the above inhibitory chemicals (hydrolysis products), these chemicals were added to the fermentation medium. In our previous studies on butanol production using *C. acetobutylicum* P262 [29] and *C. beijerinckii* BA101 [28,30], it has been demonstrated that salts inhibit butanol fermentation. However, in the present studies, we were unable to quantify the extent of inhibition caused by salts present in the system. The effect of feeding the fed-batch reactor with WSH rather than sugar solution as in the present studies is under investigation. Such fed-batch systems will reveal whether WSHs contain any inhibitory components.

4. Conclusions

These studies have demonstrated that simultaneous hydrolysis of WS and fermentation to butanol is possible with 100% hydrolysis (to the extent achievable) to simple sugars. For these studies, a fed-batch reactor of *C. beijerinckii* P260 was operated. In addition to WS, the reactor was fed with a sugar solution containing glucose, xylose, arabinose, galactose, and mannose; monomeric sugars contained in WS. The culture utilized all the sugar components. It was noticed that near the end of fermentation (286–533 h), the culture experienced difficulties in utilizing xylose. In these studies, as a result of regular feed to the reactor, ABE productivity was improved by 16%. In our previous experiment on simultaneous saccharification of WS and fermentation to butanol, a productivity of $0.31\,g\,L^{-1}\,h^{-1}$ was observed, while in the present studies, a productivity of $0.36\,g\,L^{-1}\,h^{-1}$ was observed. It should be noted that a maximum productivity of $0.77\,g\,L^{-1}\,h^{-1}$ was observed when the culture was most active. During these studies, an ABE yield of 0.44 was obtained. The fed-batch fermentation was operated for 533 h. It should be noted that *C. beijerinckii* P260 can be used to produce butanol from WS in integrated fermentations.

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